

Mechanical Dechoriation of Fertilized Eggs for Experimental Embryology in the Medaka

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ABSTRACT—Naked developing zygotes of the medaka (*Oryzias latipes*) were easily obtained by cutting off part of the chorion of the vegetal pole area with scissors. A key point for this technique was the use of just-ovulated eggs before hardening of the chorion. When unfertilized eggs were inseminated after partial dechoriation, one spermatozoon penetrated and triggered the exocytosis of cortical alveoli at the animal pole. In these eggs, supernumerary spermatozoa also penetrated and transformed into pronuclei in the cortical cytoplasm of the vegetal pole region where the chorion was absent. The polyspermic egg proper spontaneously exited the chorion into sterile saline through the opening made by the cut. Most of these naked eggs developed normally, since the male pronuclei in the vegetal pole region did not contribute to syngamy. Dechorionated eggs with no surplus spermatozoa were obtained by inseminating eggs, rinsing them briefly in 10⁻³% SDS to kill sperm in the medium, and removing the vegetal pole region of the chorion when the fertilized egg had begun to undergo cortical exocytosis at the animal pole; such eggs also became spontaneously naked in saline. Denuded embryos developed normally to fry in sterile saline supplemented with 4 µg/ml gentamicin and 6% polyethylene glycol (M_r 1,540). Surgically operated embryos also developed to fry in the same medium. The present convenient technique for dechoriation and incubation of denuded eggs seems to be suitable for experimental embryology in the medaka.

INTRODUCTION

In teleostean fishes, the embryos develop in the perivitelline fluid within a thick, tough egg membrane, the chorion [cf. 1]. The chorion, with its various elaborate ornamentations provides (1) a block to pathological polyspermy; (2) protection from bacteria, some harmful substances and mechanical shocks; and (3) a means of attaching the fragile developing embryos to aquatic vegetation. The chorion is digested by a hatching enzyme released by the prehatching embryo.

Various surgical operations on embryos are sometimes necessary when analysing the mechanisms of fish development [9-11]. In teleostean embryos, such microsurgical operations require

removal of the chorion. However, the chorion of the medaka egg becomes so hard after fertilization that it cannot be penetrated with a fine glass needle or micropipette. Moreover, because denuded embryos are susceptible to direct invasion by bacteria in the external medium, a sterile medium that will support normal embryonic development must be provided that will both substitute for the chorion and the perivitelline fluid, which may provide nutrients.

Dechoriation of medaka eggs has been performed by digestion with the hatching enzyme or with commercial proteolytic enzymes [12, 13]. The enzymatic removal of the chorion is accompanied by numerous difficulties, including the tedious preparation of hatching enzyme from prehatching embryos, control of digestion time, mechanical manipulation to remove the undigested outermost layer of the chorion, and the effects of the enzymatic treatment on the embryos to be used for experiments. In a preliminary study, we tried to

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establish a convenient method for removal of the chorion during fertilization [6].

The present paper introduces a convenient technique to mechanically denude medaka embryos without the use of any chemicals. This technique is applicable for all investigators, provided the ovulation time of the fish can be controlled by artificial circadian illumination. Embryos denuded by this technique normally developed to fry in sterile saline supplemented with antibiotics.

MATERIALS AND METHODS

Preparation and manipulation of gametes

Medakas, *Oryzias latipes*, of the d-rR strain (six-inbred: genotype bbrr, bbRr) and the wild type (two-inbred: genotype BBRR) that had been bred in our laboratory were used in the present study. They were kept under artificial conditions (14 hr light, 10 hr darkness; temp. 26–28°C) suitable for reproduction. Under these conditions, ovulation was observed about one hour before the onset of illumination [4]. Females were laparotomized after their brains were pithed. The ovaries with freshly ovulated eggs were removed from the body cavities and kept before use in a sterile saline formulated for medaka oocytes (SMO-saline: NaCl, 650 mg; KCl, 40 mg; CaCl₂·H₂O, 15 mg; MgSO₄·7H₂O, 15 mg; NaHCO₃, 100 mg; phenol red, 1.5 mg; in 100 ml of distilled water with 5 mM HEPES-HCl, pH 9, [5]) at 10–15°C. Yamamoto's saline [14] was not suitable for the present experiment because it induced gradual hardening of the chorion with time. The SMO-saline was previously sterilized by filtration through a microporous filter (pore size 0.20 µm; Advantec, Tokyo). All eggs were used within 2 hr after ovulation, since the chorion hardens slightly in overripe eggs, making partial excision of the chorion more difficult; even when a portion of the chorion could be successfully cut off with scissors, the egg proper was easily injured during manipulation by the sharp edges of the pore cut in the chorion. Consequently, when such overripe eggs showing exocytosis of a few cortical alveoli were used, a high percentage of successful dechorionations was not usually achieved.

Dechorionation was carried out with the eggs in chilled SMO-saline (10–15°C) in a Petri dish (70 mm diameter). Fine watchmaker forceps and small scissors (the blades, 0.6 mm) were used as the egg were viewed through a binocular microscope (×20). Under a binocular microscope, attaching filaments on the chorion at the vegetal pole area of the unfertilized egg were pulled with forceps in SMO-saline while the vitellus within the chorion was held back by open scissors so only the stretched part of the chorion at the vegetal pole region was cut off with the fine scissors, as described in the previous report (Iwamatsu, 1983). At this step of dechorionation, denuded eggs (the vitellus) could be obtained by carefully squeezing the vitellus out of the chorion into SMO through the large pore (200–400 µm in diameter).

A sperm suspension for insemination was prepared by releasing spermatozoa from the testes into saline. Testes were obtained from mature males by the same procedure used to obtain the ovaries. The volume of sperm suspension (1–2 × 10⁷ sperm/ml) in each watch glass (50 mm in diameter) was about 0.5–1 ml.

Insemination before dechorionation was carried out by immersing an ovary containing freshly ovulated eggs within the ovarian lumen into the sperm suspension and exposing eggs to spermatozoa by making several tears in the ovarian sac. After the eggs were rinsed once in SMO-saline containing 10⁻³% SDS to kill spermatozoa, the eggs were transferred into chilled SMO-saline and a small piece of the chorion was removed with scissors.

Injection of isolated cells into denuded eggs

Microinjection of cells from wild-type embryos into late morulae of the d-rR strain was performed by positioning the embryos in a corner of a triangular agar (2%) well made in a small Petri dish containing SMO-saline (agar dish, Fig. 1). A micromanipulator (Narishige, Tokyo) equipped with a syringe for positive pressure was used. Cells of the naked late morulae of the wild-type donor were isolated by a sterile disposable scalpel in SMO-saline in an agar dish. A micropipette with a large bore (tip diameter, ca. 10 µm) was used to transplant isolated cells into developing embryos.

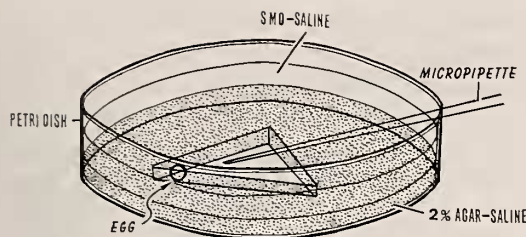


FIG. 1. Diagram of microinjection of blastomeres into medaka embryos using a micropipette held in a micromanipulator.

The micropipette was loaded with paraffin oil, then the cells to be transplanted were drawn in, and finally a small amount of the oil was drawn in to avoid a leakage of cells from the micropipette.

Cultivation of naked eggs

The culture medium used (GP-saline) was SMO-saline containing $4 \mu\text{g/ml}$ gentamicin and 6% polyethylene glycol (M_r 1,540), adjusted to pH 7.4 with 1 N NaHCO_3 . Denuded eggs were rinsed once in culture medium before they were carefully transferred with an autoclaved pipette into another agar dish containing GP-saline. Eggs that cleaved

within 2 hr after insemination were transferred and incubated in a fresh agar dish containing GP-saline (27°C , Fig. 2), which was exchanged every day. The agar dish was placed on an agitator (agitation 160 rpm; micro-mixer EM 33, Taitec, Tokyo) during incubation.

RESULTS AND DISCUSSION

Insemination and development of eggs lacking part of the chorion

Eggs partially dechorionated were transferred into fresh SMO-saline in an agar dish. The vitelline surface at the dechorionated part of the egg extruded from the pore of the chorion. These eggs were immersed into a sperm suspension in an agar-coated watch glass for about 30 sec (24°C) and then transferred into fresh SMO-saline in an agar dish. The cortical reaction was observed beginning at the animal pole region in most eggs, although many spermatozoa could also enter the extruded cortical cytoplasm at the vegetal pole region where the chorion was absent (Table 1). Only one spermatozoon was found in the cortical

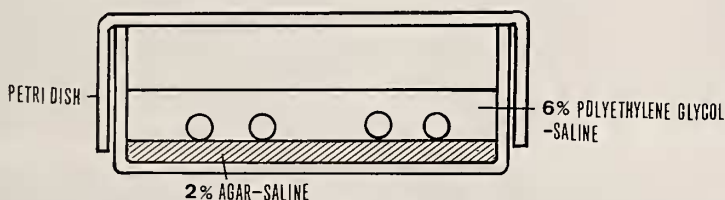


FIG. 2. Incubation of denuded eggs in sterile saline containing 6% polyethylene glycol in an agar-coated, autoclaved Petri dish.

TABLE 1. Sperm penetration in medaka eggs partially dechorionated before and after insemination

Insemination time	No. of eggs examined	No. of spermatozoa penetrated at Animal pole region	Vegetal pole region ¹
Before dechoriation			
30 sec	15	1±0 (1±0)	1.1±0.3 (0)
After dechoriation			
30 sec	15	1±0 (1±0)	6.9±1.6 (3.5±1.1)
20 min	10	1±0 ² (1±0)	22.1±6.3 (7.0±3.2)

¹ This region was dechorionated.

² In one case, two spermatozoa were found at the animal pole in an egg. All eggs were further incubated for 20 min after insemination of 30 sec or 20 min and fixed with glutaraldehyde for examination of the nucleus. The numbers in parentheses indicate the mean number of pronuclei.

cytoplasm at the animal pole region facing the inner opening of the micropylar canal.

Twenty minutes after insemination (sperm con. $1.5 \times 10^7/\text{ml}$) for 30 sec, the second polar body had been formed and the male and female pronuclei could be seen each other at the intervals of $120 \pm 22 \mu\text{m}$ in the blastodisc in all 15 eggs examined (Table 1). The mean number of surplus spermatozoa incorporated into the dechorionated region of the vegetal pole area was 7 (including 4 pronuclei) at 20 min after a 30-sec insemination.

Fifty-two eggs undergoing exocytosis at the animal pole region after insemination for 30 sec were transferred and kept in SMO-saline in an agar dish. The vitellus exited the chorion into saline by itself (Fig. 3), probably due to high

osmotic and hydrostatic pressure within the perivitelline space. The time required for "self-denuding", about 3 hr, was independent of temperature over the range $13\text{--}25^\circ\text{C}$. As seen in Fig. 4, forceps were used to carefully squeeze the vitellus out of the chorion through a large pore, when denuded eggs were needed for an experiment at the stage of blastodisc formation. Forty-eight denuded eggs (92%) developed to normal morulae. Forty of these eggs (77%) were allowed to develop to fry via normal developmental processes (Fig. 4f-h) in GP-saline (27°C) in an agar dish. The hatching enzyme was released from the hatching glands, and swimming movement was observed about 9 days after fertilization. It is likely that embryos may simultaneously acquire the capability

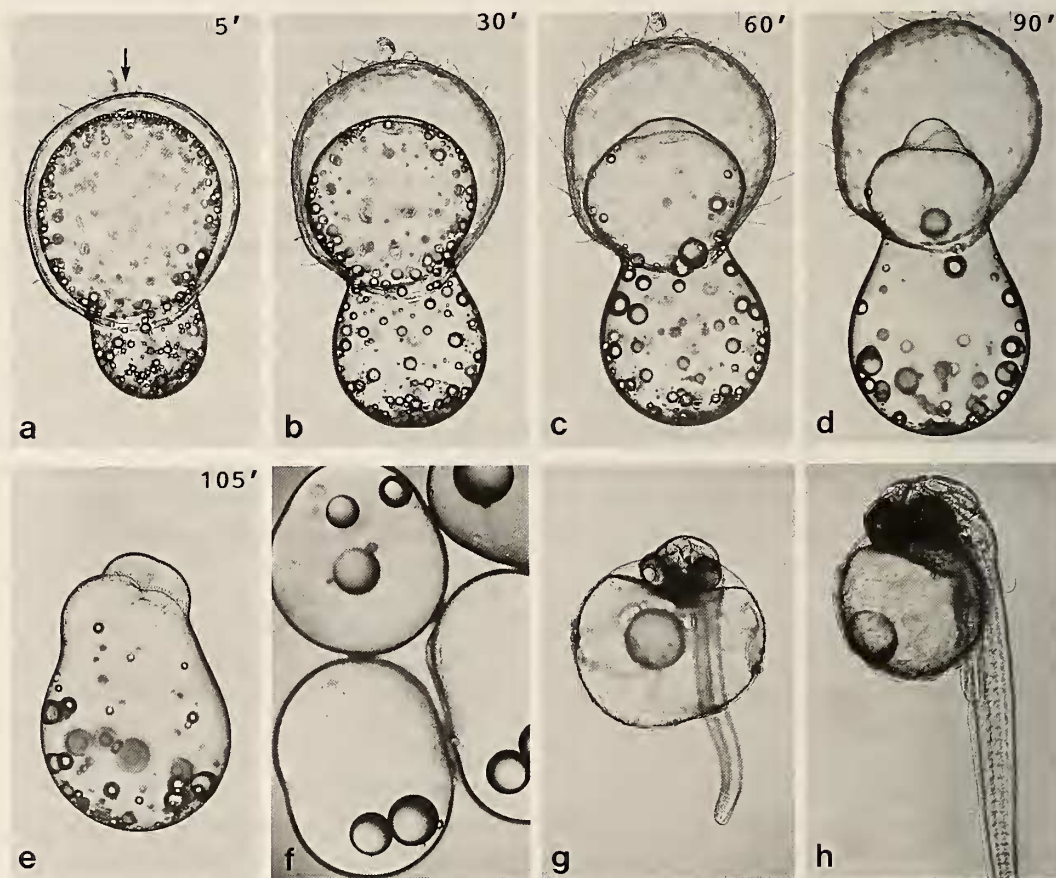


FIG. 3. Denuding and development of naked embryos. When part of the vegetal hemisphere of the chorion was cut off, the vitellus gradually extruded itself out of the chorion through the resulting cut pore (a-e, 24°C). Denuded embryos developed to fry via normal developmental processes (f, gastrulae; g, 3 day-embryo; h, 7 day-embryo, 27°C). a-f, $\times 30$; g-h, $\times 28$. An arrow in 3a indicates the micropyle.

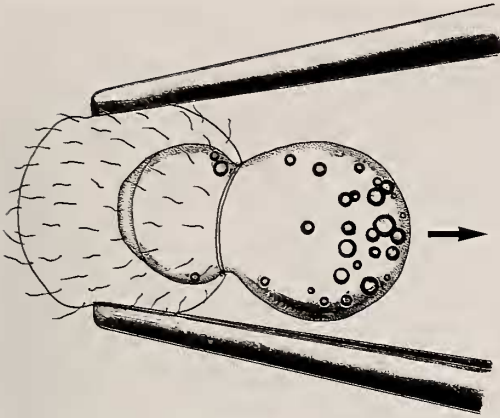


FIG. 4. Method for squeezing the vitellus out of the chorion. The vitellus is squeezed out of the chorion through the large pore by pressure on the opposite side of the chorion.

for such movement and the ability to release the hatching enzyme from hatching glands.

When 10 eggs were inseminated for 20 min and incubated further for 20 min, one supernumerary spermatozoon was found as a compact sperm head on the surface of the blastodisc in only one egg. This means that only one spermatozoon can attach to the egg plasma membrane at the animal pole region by passing through the micropylar canal; this canal is completely occluded by 5 min after insemination [8]. The mean number of spermatozoa incorporated into the dechorionated region

following 20 min of insemination was 22 (including 7 pronuclei). At the animal pole of these eggs, two gamete nuclei was observed joining each other. The supernumerary sperm nuclei in the vegetal pole area did not contributed to syngamy, because they failed to reach the animal pole region by contractile movements of cortical cytoplasm, as reported for the migration of the female nucleus [7]. Fourteen denuded eggs (78%) activated following 20 min of insemination developed normally.

Dechoriation following insemination and development of the denuded eggs

An ovary with freshly ovulated eggs was immersed in a sperm suspension in a watch glass, and each egg was immediately exposed to spermatozoa by tearing several portions of the ovarian sac with a pair of fine forceps. As soon as exocytosis began at the animal pole region after 10–20 sec of exposure to sperm, the eggs were quickly rinsed in SMO-saline containing $10^{-3}\%$ SDS and transferred into 30 ml of SMO-saline in a Petri dish (70 mm in diameter). Before hardening of the chorion began, part of the chorion was immediately cut off with scissors, using the same procedure for dechoriation as that used for unfertilized eggs, as described above (Fig. 5). Preliminary observation of sperm penetration into the dechorionated part of the cortical ooplasm was carried out on these eggs fixed with 5% glutaraldehyde (pH 7.4) 30 min

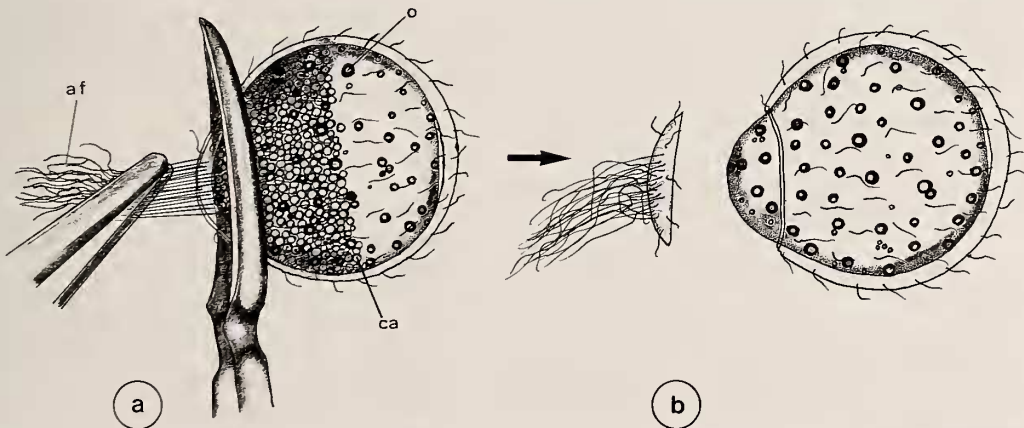


FIG. 5. Diagrammatic representation of the procedure for mechanical removal of a part of the chorion just after the beginning of exocytosis of cortical alveoli(ca) at the animal pole region following insemination. Attaching filaments (af) on the chorion in the vegetal pole area of the egg were pulled with forceps and only the stretched portion of the chorion in the vegetal pole area was cut off with fine scissors. o, oil droplet.

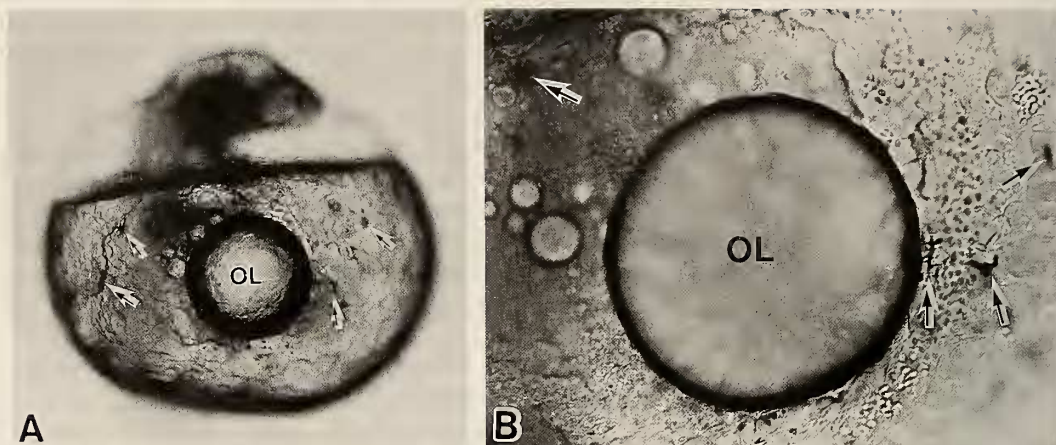


FIG. 6. A chimeric embryo of the d-rR strain medaka that has received transferred cells from a wild-type medaka 4 days after fertilization. Melanophores (arrows) are seen on the surface of the yolk sphere around the oil droplet (OL). A, $\times 55$; B, $\times 140$.

after insemination. Swollen sperm nuclei or pronuclei were not found in the vegetal pole region dechorionated (Table 1). When these 50 eggs were kept in SMO-saline in an agar dish, the vitellus exited the chorion into the saline, as shown in Fig. 2a-e. Most eggs (96%) advanced to the gastrula stage, and 88% of them developed normally to fry. This proves that the perivitelline fluid and the spherical bodies within the perivitelline space are not necessary for normal development.

As described above, the present technique for partial dechorionation before or after insemination is more convenient for obtaining naked medaka embryos than that using such proteolytic enzymes as commercial pronase and trypsin or natural hatching enzyme [2, 3, 11, 12]. Use of the enzymatic method complicates experimental schedules, because embryos must be prepared just before hatching to get the hatching enzyme.

Experimental micromanipulation of naked eggs after fertilization

Blastomeres or other cells of various adult tissues could be transferred into the vegetal pole region near the oil droplet of early developing embryos with the blastoderm by inserting a micropipette with a large bore (tip size ca. $10\ \mu\text{m}$). When blastomeres of a wild-type embryo were transplanted into the vegetal pole regions of ten

d-rR strain embryos, melanophores appeared only on the spherical yolk mass (Fig. 6) of 3 embryos. In 3 embryos the yolk sphere shrank due to leakage of the yolk from the microinjection injury into the saline during incubation. These chimeric embryos developed to morphologically normal fry in fresh sterile GP-saline. The present results indicate that naked embryos experimentally operated develop normally even out of the chorion.

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